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SHORT COMMUNICATION

Analysis of Human Immunodeficiency Virus Type 1 Promoter Insertion *in Vivo*

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Our study reports the occurrence of HIV-1 insertional activation events *in vivo*. Using a previously described PCR assay, small aliquots of broncho-alveolar lavage (BAL) cells obtained from AIDS patients were analyzed. Nine percent (5/54) of the aliquots contained proviral–host sequence transcripts indicating HIV-1 promoter insertion, whereas multiply spliced HIV-1 mRNAs were found in 28% (15/54) of the aliquots. In four of five events, insertions affect distinct cellular transcription units expressed in a T-cell line. To establish a ratio between provirus integration and promoter insertion events, an *in vitro* infection study was performed and transcripts containing HIV-1 and K-*ras* or CD4 gene sequences, respectively, were monitored. Given the randomness of retrovirus integration, 170 sense-oriented HIV integrations into these gene loci were predicted to occur. Three distinct promoter insertion events were observed, indicating that 1.8% of integrated proviruses transcribed adjacent genes. Based on this result and a mean of 257 proviral copies per 10^6 BAL cells, we would expect to observe 25 promoter insertion events in our *in vivo* study. That only five events were found may be due to the lower transcriptional activity of HIV-1 *in vivo* than that in cell cultures.

Lentiviruses are complex retroviruses, such as the human immunodeficiency viruses, which have an extended array of viral genes and an intricate regulation of gene expression. Since the HIV-1 transcriptional unit has a propensity to terminate transcription prematurely (*1*), the idea that integrated HIV-1 proviruses might transcriptionally activate adjacent host sequences seems counterintuitive. However, in a recent study using HIV-1 strain IIIB as well as a primary HIV-1 isolate, HIV-1 LTR-promoted readthrough transcripts could be demonstrated in cell cultures by PCR. Preliminary observations suggested the occurrence of insertional activation *in vivo* (*2*).

In the present study, we extend our previous observations by analyzing the occurrence and frequency of HIV-1 insertional activation events in broncho-alveolar lavage cells (BAL) obtained from 21 HIV-1-infected patients undergoing diagnostic bronchoscopy. Automated cell counts showed that BAL cells contained variable amounts of macrophages and lymphocytes (Table 1). Within 1 hr of withdrawal, cells were gently lysed and the cytoplasmic polyadenylated RNA fraction was recovered for the analysis of chimeric proviral–host sequence transcripts. Additionally, DNA was prepared from the pelleted nuclei to demonstrate the presence of proviral HIV-1 by PCR testing. Except for patient 1, all patients were PCR-positive for proviral gag and pol DNA sequences. This result is consistent with results of a recent PCR quantitation study on HIV-1 proviruses in BAL cells, where 12/14 HIV-1-infected patients were found to be PCR-positive (*3*).

The expression study of HIV-1 mRNA was performed using a previously designed and described PCR method (*2, 4*). Solid-phase cDNA pools were prepared from BAL cells by reverse transcription of cytoplasmic mRNA absorbed by oligo(dT)-linked magnetic beads (Dynabeads). cDNA pools devoid of normal HIV-1 transcripts were prepared by competitively priming the reverse transcriptase reaction with bead-linked oligo(dT) and free antisense oligonucleotides specific to the HIV LTR U3 region. Then, cDNAs primed by U3-specific primers and therefore not covalently linked to the beads were removed by melting and stringent washes, leading to the depletion of normal U3-containing HIV-1 sequences from the pool. Depending on whether competing oligonucleotide primers were included, two distinct cDNA pools resulted. The normal pool was used for analysis of multiply spliced HIV-1 mRNA, and the HIV-minus pool was used for the sensitive detection of chimeric proviral–host sequence transcripts. Both cDNA pools were PCR amplified using the same sets of primers specific to upstream HIV LTR U5 and downstream poly(dA) and poly(dA)-anchor sequences (primers M101, M102, M45, and M108) (*2*). Solid-phase cDNA pools corresponded to the mRNA content of 10^5 BAL cells. From 21 patients, a total of 108

The nucleotide sequence data reported in this article have been deposited with the GenBank Database under Accession Nos. U19177, U19178, and U19179.

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TABLE 1

Frequency of Chimeric Transcripts in Cells Obtained from Broncho-Alveolar Lavages of AIDS Patients

Patient (CDC classification)	MO (%)	Lymphocytes (%)	HIV-1 RNA PCR (pos./PCR)[a]	Chimeric PCR (pos./PCR[a], clone no.)
1 (IV)	94	5	0/2	0/2
2 (IV)	10	87	0/2	2/2 (5 and 6)[b]
3 (IV)	91	9	0/2	0/2
4 (IV)	63	25	0/2	0/2
5 (IV)	76	21	2/2	0/2
6 (IV)	14	1	0/2	0/2
7 (IV)	28	67	1/2	1/2 (4)
8 (IV)	89	13	2/2	0/2
9 (IV)	15	79	1/2	0/2
10 (III)	70	21	3/3	1/3 (2)
11 (IV)	82	18	1/3	0/3
12 (IV)	67	3	0/3	0/3
13 (IV)	90	9	1/3	0/3
14 (IV)	90	9	0/3	0/3
15 (II)	92	4	0/3	0/3
16 (IV)	73	20	0/3	0/3
17 (IV)	35	65	1/3	0/3
18 (IV)	89	9	1/3	0/3
19 (IV)	55	17	0/3	1/3 (3)
20 (IV)	15	3	0/3	0/3
21 (IV)	50	2	2/3	0/3

[a] PCR was repeatedly performed using mRNAs from aliquots of 10^5 BAL cells.

[b] Sequences of proviral–host junctions and 3′ poly(A) signals of clones 5 and 6 were already reported (clones PN5 and PN10; Ref. *2*).

such cDNA pools were analyzed using PCR, gel electrophoresis, blotting, and hybridization with oligonucleotide probes specific to LTR U5 and U3 in order to identify normal multiply spliced HIV-1 sequences and to select chimeric amplicons for sequencing. Positive results were obtained from 5/54 (9%) cDNA pools from 4 patients for the expression of chimeric and from 15/54 (28%) pools from 10 patients for multiply spliced HIV-1 sequences (Table 1 and see below). Moreover, in only 3 patients was HIV-1 expression frequent enough to be detected in every aliquot of 10^5 BAL cells tested. These findings suggest that HIV-1 insertional activation may not be a rare event considering that, under the applied conditions, PCR only detected chimeric transcripts smaller than 2–3 kb. The same size limitation prevented the detection of singly spliced or unspliced HIV-1 transcripts, leading to a lower number of cDNA pools positive for HIV-1 sequences.

Amplified fragments were isolated from the gels, cloned, and sequenced as previously described (*2*). Sequence analysis revealed five distinct chimeric provirus–host sequences (Fig. 1). Molecular clones 2, 3, and 4 stemmed from chimeric transcripts starting from the 5′ LTR, thereby containing HIV-1 leader sequences. Clones 5 and 6 exhibited nonviral sequences immediately adjacent to U5 of the 3′ LTR and could have been initiated from either the proviral 5′ or the proviral 3′ LTR. Clones 2 and 3 were spliced from the first proviral splice donor to a splice acceptor in the host sequence. Clone 4 joined the first splice donor with the proviral splice acceptor at base 5522 (numbering according to Ref. *5*), expressed HIV-1 exon 5 and spliced from the splice donor at base 5591 to an acceptor in the adjacent host sequence. The size of host-derived cellular sequences varied from 148 to 1507 bp. Northern and RT–PCR analyses determined whether these sequences belong to a host transcriptional unit. Northern analysis using a Hin-2 probe revealed two types of hybridizing mRNAs with sizes of 2.1 and 3.0 kb in TERA-1 cells (Fig. 2B). Both mRNAs are larger than the one generated by HIV-1 insertion, suggesting that integration led to truncation of the transcripts and hence the encoded peptide. Using a Hin-3 probe, Northern blot analysis showed a major band at 2.0 kb (Fig. 2B), again suggesting that the insertionally activated Hin-3 gene had been truncated. Northern analysis was negative for expressed cellular sequences contained in clones 4 and 5 when TERA-1 and H9 cells were analyzed (data not shown). Cellular sequences contained in clones 2 and 3, called Hin-2 and Hin-3 (Genbank Accession Nos. U19177 and U19178), respectively, were detected by PCR to be expressed in the neuroblastoma cell line IMR-32 (kindly provided by J. Nakagawa), the embryonal carcinoma cell line TERA-1 (ATCC), the choriocarcinoma cell line JAR (ATCC), and the T-cell line H9 (data not shown). These sequences were also detected in primary macrophages and peripheral blood mononuclear cells (data not shown). Database sequence analysis revealed that Hin-2 contains a sequence segment (nt 958–1372 of Hin-2; GenBank Accession No. U19179) that matches the sequence tag EST02615 of a fetal brain cDNA library

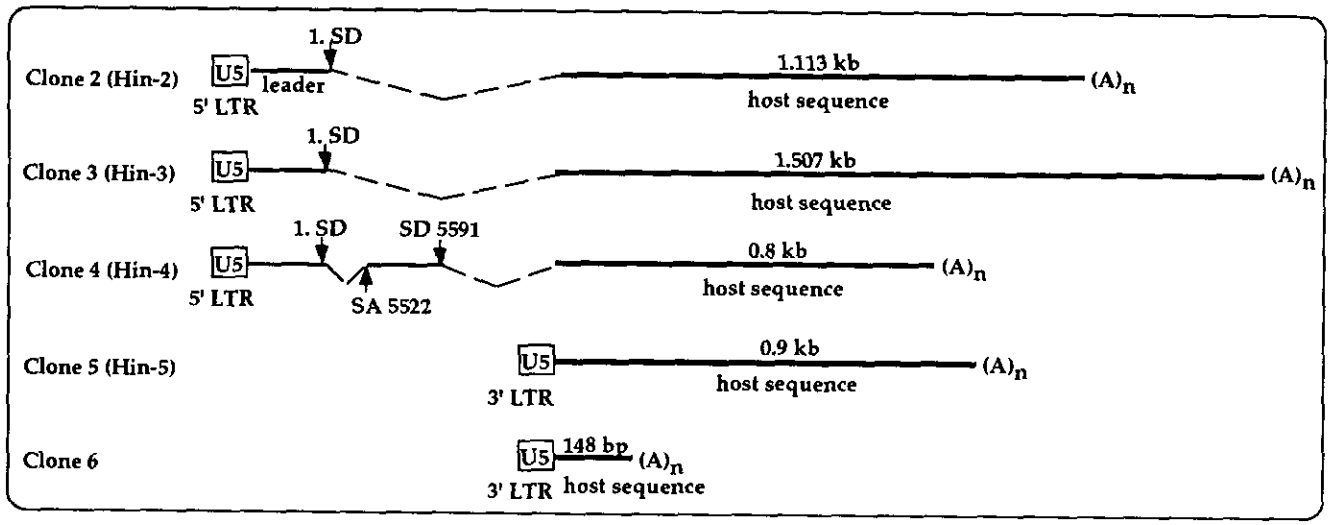

FIG. 1. Schematic diagram of the structure of five cloned amplicons representing chimeric mRNAs found in BAL cells of HIV-1-infected patients. Indicated are HIV-1 splice donors (SD) and acceptors (SA) generating the HIV-1 splice pattern and the fusion mRNA as determined by sequencing. Numbering of the proviral sequences is according to Wain-Hobson *et al.* (*5*).

in 416 of 417 bp. The finding that cellular sequences contained in clones 4 and 5, called Hin-4 and Hin-5, respectively, are expressed in uninfected H9 T-cells is shown in Fig. 2A. The amplification of cDNAs from the

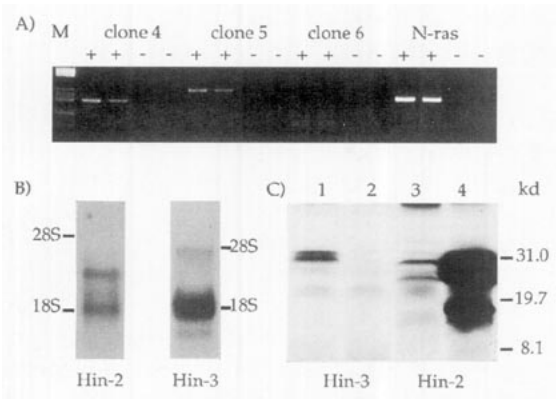

FIG. 2. Expression of chimeric provirus–host transcripts. (A) Agarose gel analysis of PCR-amplified sequences. Cytoplasmic mRNAs of H9 T-cells were analyzed by RT–PCR for the presence of transcripts matching primers specific to clone 4 (lanes 1–4; expected size 194 bp), clone 5 (lanes 5–8; 265 bp), clone 6 (lanes 9–12; 116 bp), and N-*ras* (positive control, lanes 13–16; 193 bp). Reverse transcriptase reaction was done in quadruplicate: two reactions with (+) and without (–) the addition of MoMLV reverse transcriptase. Amplification was done for 50 cycles. Lane M contains the size marker (pBR322/*Hae*III). Primers used were M679 (5′-CCCAGACTTCCTCAAAGAGTTA-3′) and M680 (5′-TCCAGAATCACCACAGACTTCG-3′) for clone 4, M681 (5′-CAAACCAGAGACTAGAGAGC-3′) and M682 (5′-TACACACTCCTGTCACTTCG-3′) for clone 5, M724 (5′-TTGGCTTCCCAAAGTGCTGG-3′) and M725 (5′-ACTATTATAGCCAGGCATTGG-3′) for clone 6, and M212 (5′-GAAATACGCCAGTACCGAAT-3′) and M100 (5′-AGACTGAAGACAGCAACAGGA-3′) for N-*ras*. (B) Northern blot analysis of Hin-2 and Hin-3 using poly(A) RNA of TERA-1 cells. The blots were hybridized to a Hin-2 or a Hin-3 antisense RNA probe. The positions of 18S and 28S ribosomal RNAs are indicated. (C) SDS–PAGE analysis of *in vitro*-transcribed and -translated chimeric mRNAs. Hin-3: lane 1, sense orientation (*s*); lane 2, antisense orientation (*as*) of the cloned insert. Hin-2: lane 3, *as*; lane 4, *s*.

T-cell line H9 using primers specific to clones 4 and 5 resulted in single fragments of the same size as previously found using genomic DNA for amplification. Since the results were dependent on prior reverse transcription, DNA contamination was ruled out. Amplification of sequences contained in clone 6 produced a weak smear of variably sized amplicons (Fig. 2A). Database search revealed that clone 6 contained a repetitive sequence element (Alu class B) which may have been primed by the downstream oligonucleotide used for PCR. We previously demonstrated an Alu repeat element in the close vicinity of the HIV-1 integration site. Stevens and Griffith (*6*) have recently suggested that repetitive DNA elements mark regions of chromatin that may act as preferred integration sites for HIV-1.

Since most splicing variants of HIV-1 do not provide a translational start site, alternative start codons in the cellular sequence must be used for translation of the residual reading frames. Hin-2 and Hin-3 exhibited predicted ORFs coding for 262 and 277 amino acid residues, respectively. The cellular sequences of clones 4 and 5 encoded distinct translational frames of 70/10/4 and 58/30/0 amino acid residues, respectively. However, only one ATG codon that could open a frame of 7 residues was present in clone 4. Therefore, analysis of *in vitro* translation was restricted to clones 2 and 3. Clones Hin-2 and 3 were recloned in vector pcDNA I/Amp (Invitrogen) in both transcriptional orientations. Then, *in vitro* transcription and translation were performed using a TNT-coupled reticulocyte lysate system according to the instructions of the supplier (Promega). [^{35}S]Methionine-labeled translation products were separated by 15% SDS–PAGE. Gels were fixed and treated for fluorography before exposure at −70°. The analysis revealed that the insertionally truncated transcripts coded for novel proteins (Fig. 2C). Hin-2, exhibiting a number of potential

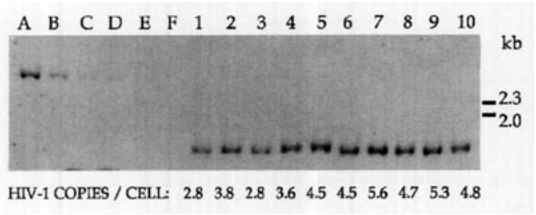

FIG. 3. Assessment of proviral copies in HIV-1-infected H9 T-cells. Southern blot and hybridization analysis of *Bgl*II-digested control DNA (lanes A–F) and DNAs extracted from 10 H9 cell cultures infected with HIV-1 IIIB for 7 days (lanes 1–10). DNAs in lanes A–E are spiked with 10, 5, 2.5, 1.25, and 0.6 pg, respectively, of linearized plasmid pGgag-1. Using an SP6 transcribed, ^{32}P-labeled RNA probe specific to HIV-1 gag, a single internal restriction fragment of 1.6 kb became apparent in DNAs of infected cells. Below, the calculated numbers of integrated proviruses per cell are indicated.

initiation codons, showed two equally strong bands of about 28.5 and 25 kDa corresponding to the predicted molecular weight of 28,290 (Met 1) and 25,094 Da (Met 26), respectively. Additional shorter peptides were probably related to the use of alternative start codons (e.g., Met 97). Hin-3 showed two bands of approximately 32 and 30 kDa. The larger corresponded to the predicted 277-amino-acid peptide of 31,507 Da. The lower band, with a calculated size of 29,520 Da, could arise from an alternative start codon (Met 17) in the same frame.

To confirm the above observations and to establish the frequency of promoter insertion, we used *in vitro* infection. The frequency of HIV-1 promoter insertion into the CD4 and K-*ras*-2 genes as well as the insertional events between HIV-1 and Hin-1 (*2*), Hin-2, and Hin-3 genes, respectively, was studied by independently infecting 10 cultures of 1.5×10^6 H9 T-cells with HIV-1 IIIB (0.5 ml frozen stock of a supernatant from a chronically infected H9 culture for 1 hr at 37°). Seven days after infection, cultures were analyzed for HIV-1 provirus integration as well as for the occurrence of chimeric transcripts consisting of HIV-1 and one of the above-mentioned genes. The number of integrated proviruses in infected H9 cells was determined by digestion of 5 μg DNA with *Bgl*II and Southern blot analysis (Fig. 3). DNA was prepared by phenol/chloroform extraction after proteinase K digestion of HIV-1-infected H9 cells in lysis buffer containing urea (Applied Biosystems). Addition of salt and ethanol allowed the preferential precipitation of high-molecular-weight DNAs, and the samples were nearly devoid of unintegrated proviruses as confirmed by Southern blot and hybridization analysis of undigested DNA samples (data not shown). Digested DNA samples were hybridized using a ^{32}P-labeled RNA probe obtained from SP6 transcribed linearized pGgag1 containing a 266-bp insert of HIV IIIB gag [nt 822–1087 (*5*)]. Signal intensities were measured by phosphorimaging (Molecular Dynamics) and compared to those of titrated plasmid pGgag1 (size: 3.3 kb). The analysis of integrated HIV-1 showed that, on average, every culture cell carried 4.2 integrated provirus copies.

PCR analysis of 3×10^6 HIV-1-infected cells corresponding to 12.6×10^6 integration events was done for chimeric transcripts using upstream primers specific to HIV-1 LTR U5 and downstream primers specific to the 3′ end of the coding region of the gene under investigation. Since the complete genomic size of the Hin genes was unknown, the arbitrarily chosen CD4 and K-*ras*-2 genes were used to determine the frequency of HIV-1 promoter insertion. They exhibit genomic sizes of 35 and 45–50 kb, respectively (*7, 8*). Since amplicons would not contain the relatively long 3′ untranslated region of these genes, PCR analysis of HIV-1 promoter insertion covered a total of about 80 kb of genomic DNA. Assuming that HIV-1 integrates randomly into proliferating T-cells (genome size: 3×10^6 kb), 340 integration events in the regions under study were predicted to occur. With regard to the host genes, 170 integrated proviruses would exhibit the correct transcriptional orientation to result in detectable readthrough transcripts. Whereas the analysis failed to detect chimeras between HIV-1 and CD4 sequences, four bands were detected in the hybridization analysis performed to identify insertional activation of a K-*ras* gene (Fig. 4A). Three of them could be cloned, and sequencing demonstrated that they represent transcripts from the K-*ras*-2 locus. Since the identity of the fourth chimeric transcript could not be established by cloning and sequencing, it was omitted from further analysis. All cloned K-*ras*-2 chimeric transcripts used the first HIV-1 splice donor. One used the splice acceptor of the K-*ras*-2 exon 2 and two used that of exon 3 (Fig. 4B). Assuming that all inserted proviruses were transcriptionally active, HIV-1 readthrough transcription was detected by this method with a frequency of 1.8%.

The second group of genes analyzed included those previously found by HIV-1 integration. No excessive number of promoter insertions was detected. HIV-1 promoter insertions could not be observed in Hin-1 and Hin-3. However, in the Hin-2 gene locus, three independent insertional activations could be demonstrated by Southern blot and oligohybridization analysis of the PCR products (Fig. 4C) and subsequent cloning and sequencing. All occurred by transcriptional readthrough starting from the HIV-1 5′ LTR promoter and using the first or a downstream splice donor to join with the Hin-2 sequence (Fig. 4D). In two cases, the splice acceptor of Hin-2 was downstream of that observed in the patient study; in the third case it was located 705 bp upstream. Sequencing of this clone revealed an extended open reading frame for Hin-2 encoding a predicted protein of at least 416 amino acid residues.

Readthrough transcripts can be expected whenever cleavage and polyadenylation of viral transcripts in the 3′ LTR are inefficient. In the case of HIV-1, transcription studies with transfected constructs showed that the HIV-1 poly(A) site is an efficient 3′ processing signal (*9*). However, proximity of strong polyadenylation signals or single base mutations in the poly(A) site could render

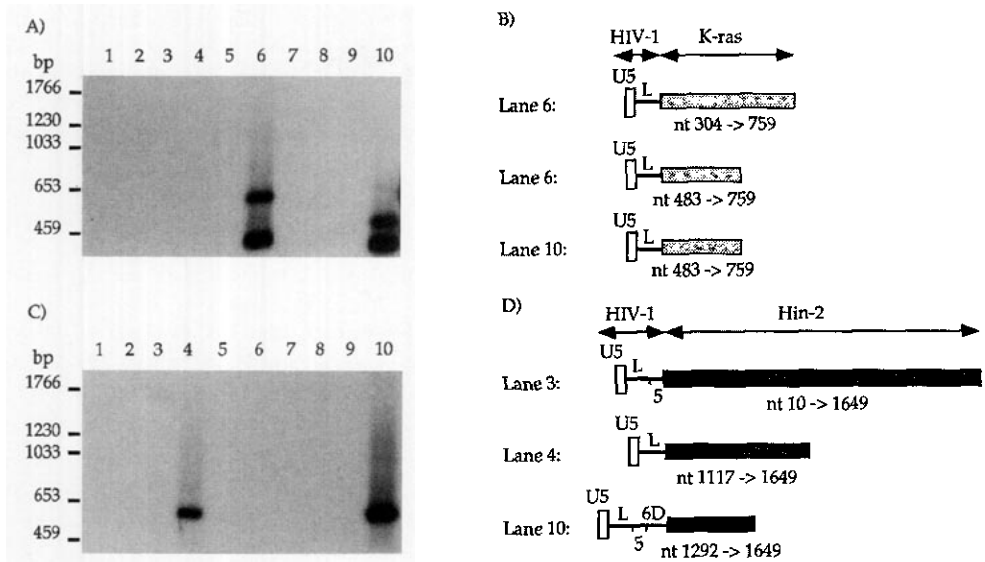

FIG. 4. Search for insertional activation of Hin-2 and K-*ras* in HIV-1-infected cell cultures. (A) Hybridization analysis of K-*ras*-2-containing amplicons obtained from the mRNAs (mRNA equivalent of 3×10^5 H9 cells each) from 10 infected H9 cell cultures (lanes 1–10) using chimeric PCR. HIV-1 U5-specific upstream primer M103 (*2*) and downstream primer specific to K-*ras* (M601, 5′-dTTACATAATTACACACTTTGTCTTTG-3′) and a ^{32}P-labeled oligoprobe (M617, 5′-dTTACCATCTTTGCTCATCTTTTC-3′) were used. (C) Hybridization analysis of chimeric Hin-2 amplicons using the ^{32}P-labeled oligoprobe M429 (5′-dGAGTATACAACAACATG-3′). Primers used for amplification of chimeras between HIV-1 and Hin-2 were 5′ oligonucleotide M103 and 3′ oligonucleotide M440 (5′-dGTCAGCAAACACCTGAACCTG-3′). (B and D) Schematic representations of the structure of cloned and sequenced chimeric amplicons. Viral sequences contained U5 from the 5′ LTR (open boxes), the leader sequence (L), exon 5 of HIV-1 in two isolates, and exon 6D in one. [Exon designation according to Schwartz *et al.* (*15*).] Host sequences (filled boxes) of the amplicons exhibited variable parts of K-*ras*-2 (C) or Hin-2 (D). Numbers correspond to nucleotides (nt) in the K-*ras*-2 cDNA (M54968; Hskrasm.pri) or Hin-2 cDNA (GenBank Accession No. U19179).

the site inefficient and readthrough transcription would become detectable (*10*). It is not known whether mutations in the poly(A) site of the identified chimeric transcripts played a causative role because, due to technical reasons, the poly(A) site was not contained in the amplicons produced by chimeric PCR and could not be analyzed.

We asked whether the frequency of chimeric transcripts found *in vitro* is consistent with the data obtained from the patient study. From 21 patients, a total of 5.4×10^6 BAL cells were analyzed. Assuming an average of 257 proviral copies per 10^6 BAL cells (*3*), we would expect to find 25 ± 9.8 insertional activations of host sequences. In contrast, we found 5 insertional activations. This discrepancy between *in vitro* and *in vivo* results may have several explanations. Sampling and amplification procedures differed in that processing of *in vitro* specimens occurred faster and the stability of chimeric mRNAs may have differed under these conditions. More importantly, the generation of chimeric transcripts may be dependent on the transcriptional activity of HIV-1 proviruses, which is lower *in vivo* than in *in vitro*. In recent reports using sensitive *in situ* PCR methods, 1–10% of infected cells were found to express HIV-1 transcripts (*11, 12*).

In summary, our study demonstrates that HIV-1 promoter insertion occurs *in vivo* with a substantial frequency. However, consequences for the viral–host interrelationship are not yet elucidated. HIV-1 insertion may truncate the coding region of host genes without preventing the expression of peptides from alternative translational start sites. We speculate that some of these truncated peptides would behave as autoantigens, thereby supporting the chronic immune activation observed during HIV infection. Furthermore, a well-known risk associated with retrovirus promoter insertion is tumorigenesis. The resulting tumors are characterized by a common clonal retroviral integration site (for review, see Ref. *13*). A recent study suggests such a site to be present in HIV-1-associated T-cell or mixed immunophenotype lymphomas (*14*). Therefore, the study of *cis*-acting effects of HIV-1 regulatory regions may be useful in evaluating the risks of HIV-1 vector-based gene therapy. The relatively frequent occurrence of promoter insertion which we report suggests that modification and enforcement of the poly(A) processing signals in the 3′ LTR of such vectors would be necessary.

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